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ORIGINAL ARTICLE

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Abstract

Current histological grading recommendations for isocitrate dehydrogenase (IDH)-mutant astrocytoma are imprecise and not reliably predictive of patient outcome, while somatic copy number alterations are emerging as important prognostic biomarkers. One explanation for this relative underperformance of histological grading is that current criteria to distinguish World Health Organization (WHO) grade III anaplastic astrocytomas from lower-grade diffuse astrocytomas (WHO grade II) are vague ("increased mitotic activity"). This qualitative approach ensures diagnostic uncertainty and a broad "gray zone" where both diffuse and anaplastic designations can reasonably be assigned. Thus, we hypothesized that interobserver variability and lack of defined mitotic thresholds for IDH-mutant astrocytomas underlies poor predictive accuracy of current histologic grading approaches. To test this hypothesis, we quantified total mitotic figures and maximum mitotic activity per 10 high-powered fields in an institutional cohort of IDH-mutant astrocytomas. In our cohort, there was no mitotic activity threshold that was reflective of progression-free or overall survival (OS). Furthermore, in a multivariate Cox regression model consisting of mitotic activity, molecular markers, and clinical characteristics, only *CDKN2A* homozygous deletion was identified as a relevant variant for poor OS. We conclude that lack of defined mitotic figure thresholds may not contribute to underperformance of histological grading for IDH-mutant astrocytomas.

Key Words: Astrocytoma, Glioma, Histopathology, Isocitrate dehydrogenase (IDH), Mitosis.

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INTRODUCTION

The World Health Organization (WHO) introduced a classification system of diffuse gliomas in 2016 that integrates traditional histopathological features with specific molecular alterations to define "integrated" diagnostic entities (1). The molecular drivers of this diffuse glioma classification system is driven broadly by point mutations in exon 4 of *isocitrate dehydrogenase 1/2* (*IDH1/2*) and codeletion of whole chromosome arms 1p and 19q. Integrating these 2 molecular alterations into the historical classification system of diffuse glioma has yielded a better and more reproducible predictor of clinical outcome than traditional histopathology alone (1, 2). Although this integrated classification system has much improved reproducibility and concurrence across diagnosticians, identifying tumors with aggressive or malignant potential in the context of IDH-mutational status remains challenging, especially for IDH-mutant astrocytic gliomas. This histopathological grading scheme for differentiating diffuse astrocytoma (WHO grade II) from anaplastic astrocytoma (WHO grade III) ("increased mitotic activity") was not updated with the integrated classification system, and does not take into account IDH-mutational status.

For IDH-mutant astrocytic gliomas, differentiation between diffuse astrocytoma (WHO grade II) and anaplastic astrocytoma (WHO grade III) based on histological features appears to have limited, if any, utility in discriminating outcomes amongst these tumors (3–5). The historical utility of the current histologic grading approach is most likely due to the inclusion of poorly surviving IDH-wildtype astrocytomas that tended to have higher histological grade. With the new integrated approach, IDH-mutational status provides effective differentiation of generally more (IDH-wildtype) or less (IDH-mutant) aggressive astrocytomas, with a broad range in each group in which histological grading may provide additional predictive value. However, there are currently no diagnostic criteria that specifically separate IDH-mutant or IDH-wildtype tumors into subgroups with predicted survival, and broadly applied criteria are generally not helpful.

In comparison to histological features, there are emerging studies demonstrating the utility of certain molecular markers reflective of aggressive behavior within IDH-mutant astrocytomas. Our group (5, 6), and others (7–9), have demonstrated that a handful of molecular somatic copy number

alterations (SCNAs) portend survival within IDH-mutant astrocytic gliomas. These SCNAs include *CDKN2A* deletion (5, 6, 8, 10), total copy number load (8, 11), chromosome 12p gain/*CDK4* amplification (6, 9, 10), and loss of chromosome 14 (5, 6, 9). However, these SCNAs, or other molecular markers, are not widely available for diagnostic neuropathology practice, so there is still a pressing need for a predictive histologic variable beyond "increased mitotic activity" for grading for IDH-mutant astrocytomas.

As has been shown in some other tumor types (4, 12–15), a rational and practical mitotic index threshold for grading IDH-mutant astrocytomas could be effective for patient risk-stratification. Clear mitotic index cutoffs for predicting survival for IDH-mutant astrocytomas would guide reproducible histological grading and improve diagnostic practice and thus patient care, especially in resource-limited environments. Furthermore, clear histological guidelines could help to reduce astrocytoma grading variability among pathologists (16). We hypothesized that quantitative thresholds for mitotic activity would improve predictive accuracy and could therefore be useful for histopathological grading of IDH-mutant astrocytomas. To test this hypothesis, we reviewed a consecutive institutional cohort of newly diagnosed resected IDH-mutant astrocytomas and systematically evaluated various quantitative mitotic indices to determine if specific thresholds could predict clinical outcome, and thus help to establish concrete guidelines for practical routine surgical neuropathology practice.

MATERIALS AND METHODS

Case Selection

The use of human subject material was performed in accordance with the World Medical Association Declaration of Helsinki and with the approval of the University of Washington Institutional Review Board. Institutional records were queried to identify newly diagnosed WHO grade II and III diffuse gliomas occurring between 2000 and 2010 as previously described (5). Chart review was performed to ascertain extent of clinical follow up and additional clinical characteristics. Cases included in the study had clinical follow up of at least 5 years, or recurrence or death if sooner. The diagnosis of a WHO grade II or III diffuse glioma was confirmed by slide review of a board-certified neuropathologist (P.J.C.). Cases with additional tissue material available underwent molecular analysis for classification according to current WHO guidelines.

Diffuse Glioma Classification

DNA was extracted from formalin-fixed paraffin-embedded (FFPE) tissue blocks using the QIAamp DNA FFPE Tissue Kit (Qiagen Inc., Germantown, MD) and underwent Qubit fluorometric quantitation (Invitrogen, Carlsbad, CA). Each tissue block tested had tumor representing at least 70% of the tissue present. Quality control of the DNA was performed using the Infinium FFPE QC Kit (Illumina, San Diego, CA) according to the manufacturer's protocol. Bisulfate conversion of the DNA using the EZ-DNA Methylation Kit (Zymo Research, Irvine, CA) was followed by use of the FFPE Restoration Kit (Illumina) according to the manufacturer's protocol and included the Illumina "Alternative Incubation Conditions" modification to the conversion protocol. The DNA was then processed for genome-wide methylation analysis using the InfiniumR EPIC Methylation Array (Illumina). IDAT files were uploaded to the dkfz molecular neuropathology website (https://www.molecularneuropathology.org) and analyzed (classifier v11b4 version 2.0) to classify diffuse gliomas into 1 of the 3 following categories: Astrocytoma IDH-wildtype, astrocytoma IDH-mutant, and oligodendroglioma IDH-mutant and 1p/19q-codeleted (17). Additionally, this analysis was used to determine methylation status for the *O-6-methylguanine-DNA methyltransferase* (*MGMT*) promoter.

Histological Slide Review

Routine diagnostic hematoxylin and eosin (H&E)-stained slides for each molecularly classified IDH-mutant astrocytoma (n = 34) were reviewed for mitotic figures independently by either RAY, TM, or LL. All tissue on every slide for each resection specimen was scanned with a 40× objective to quantify total mitotic counts (upper threshold cap of >20 mitoses per specimen) and maximum mitoses per 10 consecutive high-power fields (HPF). A single HPF is defined in this study of having a field diameter of 0.55 mm and an area of 0.24 mm^2. The upper limit cap for total mitotic figures was the point where reviewers ceased counting mitotic figures per specimen. For slide review, there was no upper limit cap for counting mitoses per 10 HPF. Reviewers were blinded to the original neuropathological diagnosis. At least 2 reviewers independently assessed all slides of 9 of 34 (26.5%) astrocytomas (Supplementary Data Table S1) to determine interobserver reliability.

Statistics

Interreviewer agreement for mitotic thresholds was calculated to determine Cohen's kappa coefficient (18, 19) for 3 separate threshold tiers: Total mitotic figures (<20 vs ≥20), maximum mitotic figures per 10 HPF (<3 vs ≥3), and maximum mitotic figures per 10 HPF (<6 vs ≥6). Cohen's kappa coefficient determination and Kaplan-Meier analysis for progression-free and overall survival (OS) were performed using GraphPad Prism software (Version 7.02, https://www.graphpad.com/scientific-software/prism). P values were determined by Cox proportional hazards regression. Time-dependent receiver operating characteristic (ROC) estimation for censored survival data was performed using R software (Version 3.4.4, *R*Project for Statistical Computing, http://www.r-project.org/) with the "survivalROC" package (https://cran.r-project.org/package=survivalROC); median survival times used as cutoffs. Multivariate Cox proportional hazards models with indicated variables were applied utilizing SPSS statistical software Version 25.0 (IBM, Armonk, NY).

RESULTS

Review of our institutional files initially identified 178 cases of newly diagnosed adult WHO grade II and III

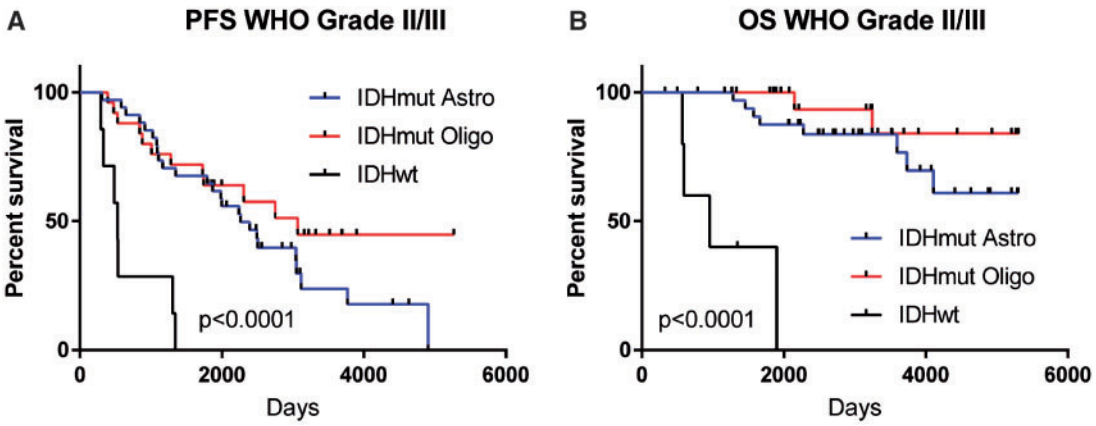

FIGURE 1. Kaplan-Meier survival curves for entire cohort of World Health Organization (WHO) grade II and III diffuse gliomas. **(A)** Progression-free survival (PFS) and **(B)** overall survival (OS) for all diffuse gliomas based upon major classification subtypes (Astro = astrocytoma; Oligo = oligodendroglioma, Mut = mutant; WT = wildtype). P values were determined using Cox proportional hazard regression.

diffuse gliomas that were resected between the years 2000 and 2010. Of these 178 patients, 109 had documented clinical follow up of at least 5 years, if not recurrence before this time point. Pathology slides from these 109 patients were rereviewed to confirm the diagnosis of a WHO grade II or III diffuse glioma. Tissue blocks with sufficient material for molecular characterization existed for 95 of these cases. DNA was extracted from these tissue blocks, tested for DNA quality, and underwent whole-genome methylation analysis for glioma classification according to the 2016 WHO guidelines (1). A total of 66 WHO grade II and III diffuse gliomas had DNA that passed quality control and were successfully classified into the following 3 major integrated categories: Astrocytoma IDH-wildtype, WHO grade II/III (n = 7); astrocytoma IDH-mutant, WHO grade II/III (n = 34); and oligodendroglioma IDH-mutant and 1p19q-codeleted, WHO grade II/III (n = 25). Similar to other studies of WHO grade II and III diffuse gliomas (2, 20–22), IDH-wildtype astrocytomas had the worse prognosis (median progression-free survival = 532 days, median OS = 956 days), which was followed by IDH-mutant astrocytomas (median progression-free survival = 2261 days, median OS = undefined) (Fig. 1). The best prognosis was observed in oligodendroglioma IDH-mutant and 1p/19q-codeleted (median progression-free survival = 3073 days, median OS = undefined).

We chose to evaluate quantitative mitotic thresholds in relation to clinical outcome in the IDH-mutant subgroup (median age 32.5 years, range 22–55 years) to attempt to clarify the current "gray zone" for histological grading of IDH-mutant astrocytomas based upon "increased mitotic activity." Representative histological features of this cohort of IDH-mutant astrocytomas are shown in Figure 2. We quantified all original H&E resection slides for the IDH-mutant astrocytomas for mitotic activity, with moderate to very good interobserver reliability; Cohen's kappa coefficients: $\kappa = 0.546$ (moderate agreement for <20 vs ≥20 total mitotic figures), $\kappa = 1.000$ (very good agreement for <3 vs ≥3 maximum mitotic figures per 10 HPF), $\kappa = 0.571$ (moderate agreement for <6 vs ≥6 maximum mitotic figures per 10 HPF). Total mitotic figures identified per resection specimen (all slides), with an upper limit of at least 20 mitoses, were not predictive of clinical outcome for either progression-free survival (PFS) (p = 0.873) or OS (p = 0.300) based on Kaplan-Meier analysis (Fig. 3). Because total mitotic activity may theoretically be a function of the size of resection, we accounted for this by quantifying maximum mitotic figures present per 10 HPF. Aggressive clinical behavior of IDH-mutant astrocytomas was not reflected by maximum mitotic figures (thresholds of 1–6 mitotic figures per 10 HPF) for either PFS (Fig. 4) or OS (Fig. 5) by Kaplan-Meier analysis. Likewise, ROC curve analyses for both total mitotic figures per case and maximum mitotic figures per 10 HPF failed to identify an association between mitotic activity and PFS or OS (Fig. 6).

In addition to mitotic index thresholds, we sought to determine molecular and/or clinical features that may be independently associated with decreased PFS and OS in this institutional cohort. In a Cox regression model of inferior PFS (Table), we identified relevant associations of *MGMT* promoter methylation status (methylated vs unmethylated: HR = 0.52, 95% CI = 0.09–0.69, p = 0.007) and chromosome 14 loss (presence vs absence: HR = 8.00, 95% CI = 1.99–32.26, p = 0.003). There was also a trend toward an association with extent of resection (gross total vs subtotal: HR = 0.40, 95% CI = 0.15–1.03, p = 0.058). No associations were found for total mitotic figures, *CDKN2A* homozygous deletion status, patient age (age <40 vs ≥ 40 years), or Karnofsky Performance Score (KPS). Applying a similar Cox regression model to explore predictors of inferior OS, we identified *CDKN2A* deletion (homozygous deletion vs no homozygous deletion: HR = 11.76, 95% CI = 1.98–71.43, p = 0.007) as the most relevant predictor of outcome (Table). There was a trend toward inferior outcome utilizing a cut-off for total mitotic figures (≥20 vs <20: HR = 5.86, 95% CI = 0.92–37.55, p = 0.062), whereas no relevant association with OS was detected for *MGMT* promoter methylation status, loss of chromosome 14, patient age (age <40 vs ≥40 years), KPS, or extent of resection. In our Cox proportional hazards

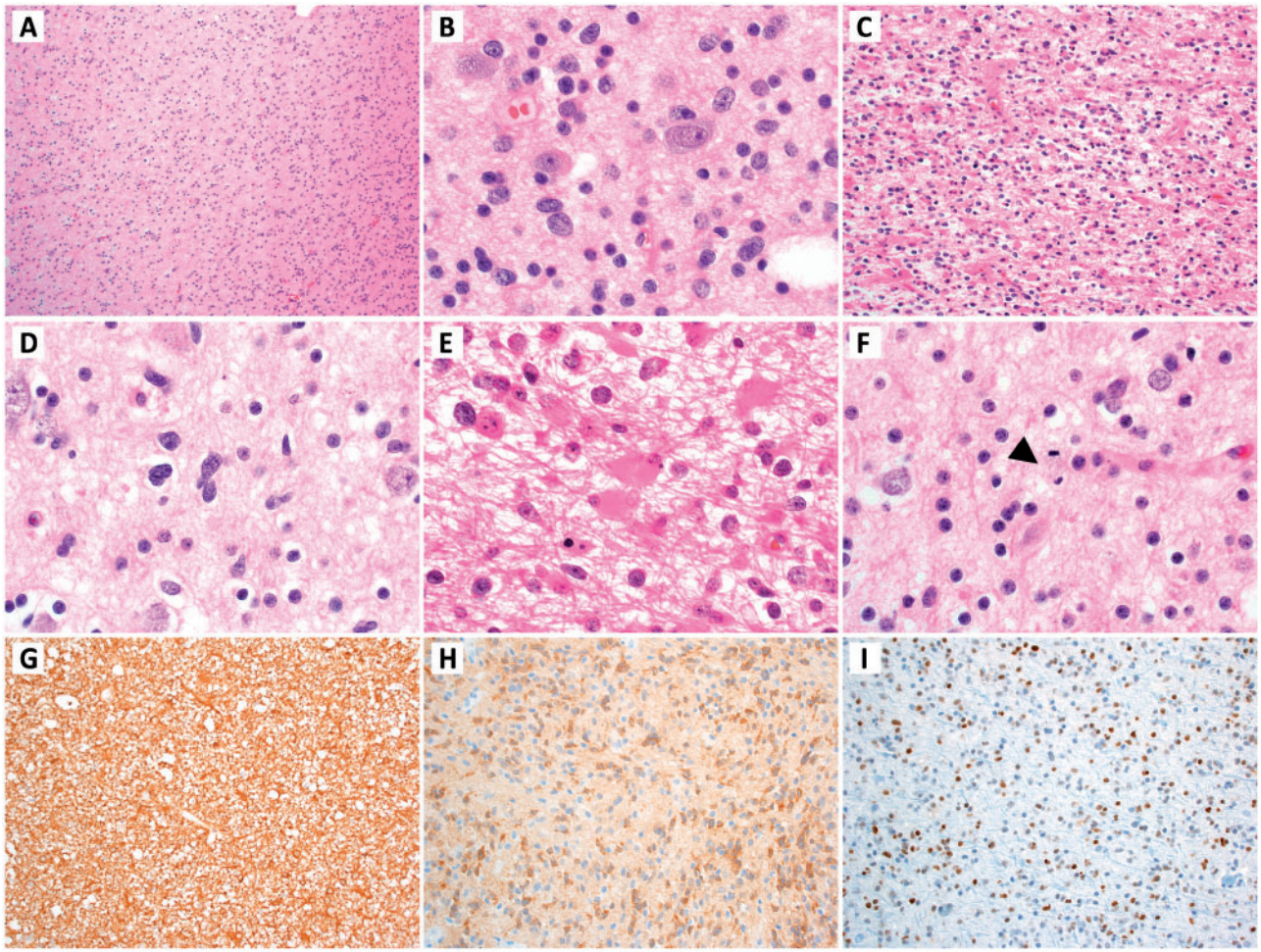

FIGURE 2. Representative histology for our institutional cohort of IDH-mutant astrocytomas. These gliomas are diffusely infiltrative into the cortex and subcortical white matter, as evidenced by **(A)** graded hypercellularity and **(B)** the presence of background cortical neurons (original magnification, 200× and 600×, respectively). **(C)** There are intra and intertumoral regions or variably increased relative hypercellularity (200× original magnification). The neoplastic astrocytic cells appear predominantly as having **(D)** fibrillary and/or **(E)** gemistocytic cytomorphologies (original magnification, 600×). **(F)** Mitotic figures (arrowhead) are variably present (600× original magnification). Immunohistochemistry shows that neoplastic cells generally have cytoplasmic reactivity for **(G)** GFAP and **(H)** IDH1-R132H mutation, as well as increased nuclear **(I)** p53 staining (original magnification, 200×).

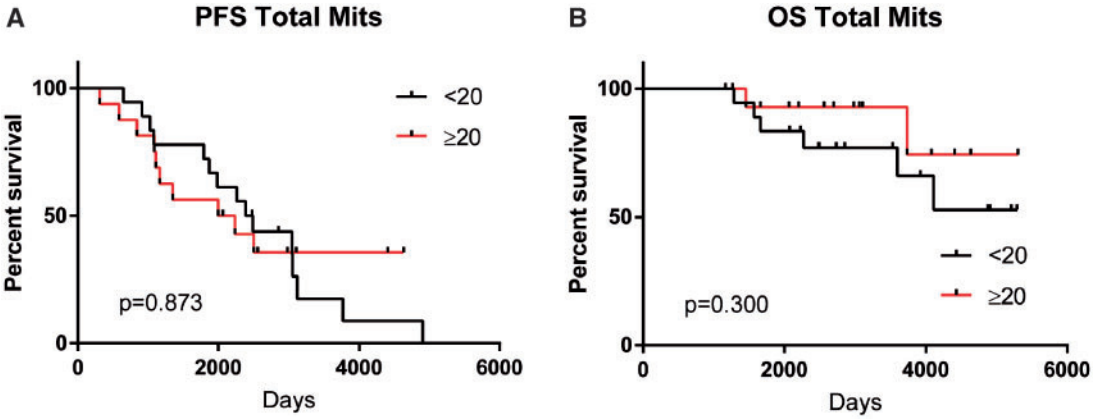

FIGURE 3. Kaplan-Meier survival curves for IDH-mutant astrocytomas stratified total counted mitotic figures. **(A)** Progression-free survival (PFS) and **(B)** overall survival (OS) do not differ between astrocytomas with <20 total mitoses versus those with 20 or more mitoses present per resection specimen. P values were determined using Cox proportional hazard regression.

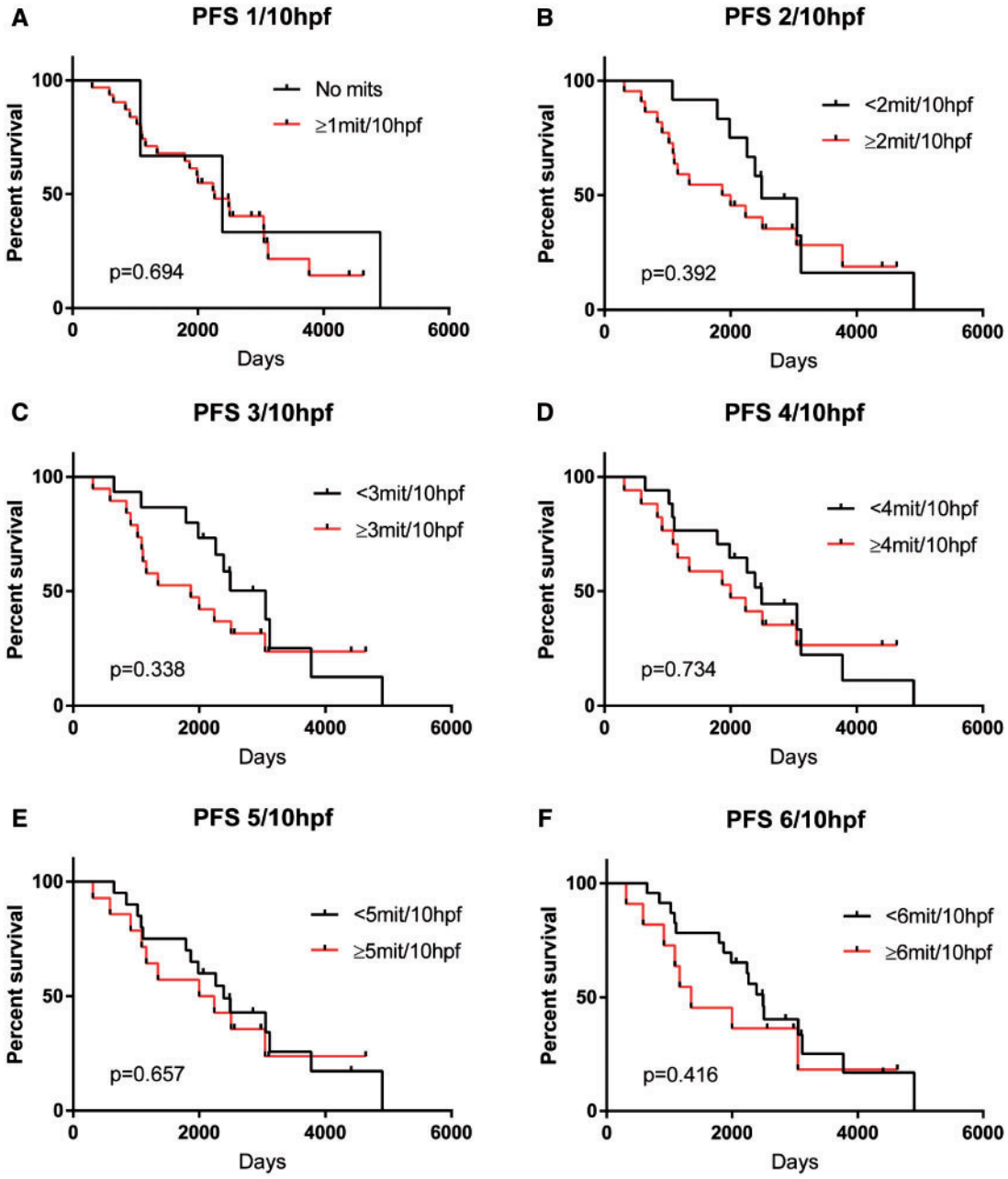

FIGURE 4. Kaplan-Meier progression-free survival (PFS) curves for IDH-mutant astrocytomas stratified by maximum number of mitoses identified per 10 high-power fields (10 HPF). Mitotic thresholds for each comparison were performed for **(A)** 1, **(B)** 2, **(C)** 3, **(D)** 4, **(E)** 5, and **(F)** 6 mitotic figures per 10 HPF. P values were determined using Cox proportional hazard regression.

model, age 40 was used as a cut-off as this has been used as an established prognostic marker for patients with lower-grade diffuse gliomas that guides risk-management and the decision of watchful waiting versus early treatment strategies (23–28). While these previously published studies may not be exactly comparable as our current study, which focuses exclusively on IDH-mutant astrocytomas (WHO grades II and III), our 40-year age cut-off allows for some meaningful comparison across these historical published reports. In addition to using age 40 years as a cut-off, we also evaluated our Cox model in relation to median age of our cohort. Replacing the 40-year cut-off for the median age (<33 vs ≥33 years) in our Cox model does not change significance of any parameters examined and itself is not associated with PFS (HR = 0.69, 95% CI = 0.24–1.96, p = 0.49) or OS (HR = 0.08, 95% CI = 0.01–1.18, p = 0.67).

DISCUSSION

Current approaches to distinguish between WHO grade II and III IDH-mutant diffuse astrocytic gliomas for clinical risk-stratification have no real prognostic significance (3, 5).

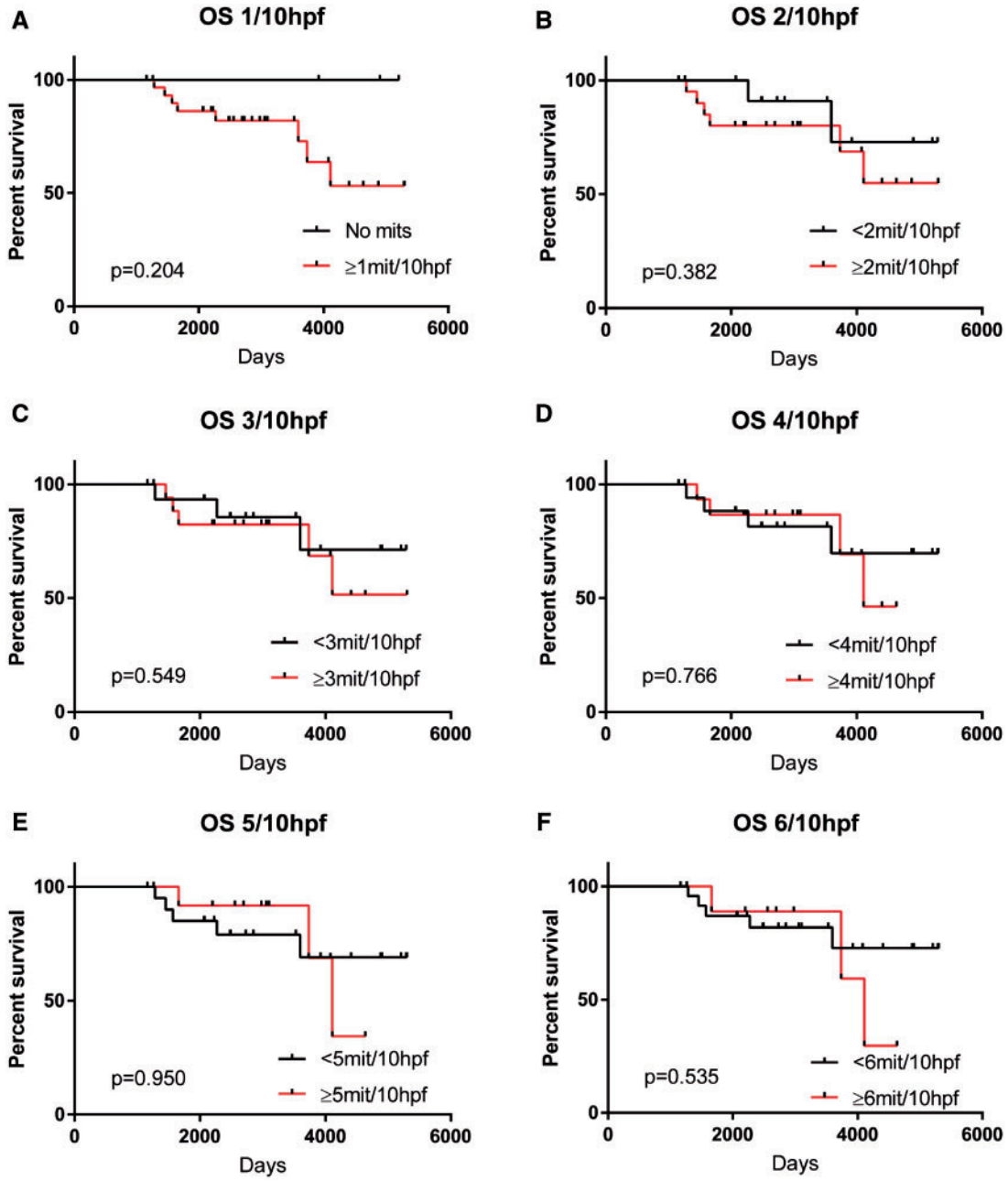

FIGURE 5. Kaplan-Meier overall survival (OS) curves for IDH-mutant astrocytomas stratified by maximum number of mitoses identified per 10 high-power fields (10 HPF). Mitotic thresholds for each comparison were performed for **(A)** 1, **(B)** 2, **(C)** 3, **(D)** 4, **(E)** 5, and **(F)** 6 mitotic figures per 10 HPF. P values were determined using Cox proportional hazard regression.

Given that the WHO histological grading scheme is based solely upon poorly defined "increased mitotic activity", which in turn contributes to increased interobserver variability (16), we sought to determine if quantitative mitotic thresholds informed by clinical outcome could be ascertained for grading classification of IDH-mutant astrocytomas. If such criteria could be established, perhaps they could be applied to future studies and compensate for previously described underperformance of histological grading strategies. However, in our cohort, predictive thresholds could not be established either for total mitotic activity or for maximum mitotic activity per 10 HPF. It is possible that our threshold cap for total mitotic activity (20 total mitotic figures per resection specimen) was too low and that a higher threshold would have provided statistically relevant predictive separation of groups. However, we evaluated each level of mitotic activity with respect to 10 HPF and see no significant trend toward separation of groups by PFS or OS with increasing mitotic activity. We interpret these

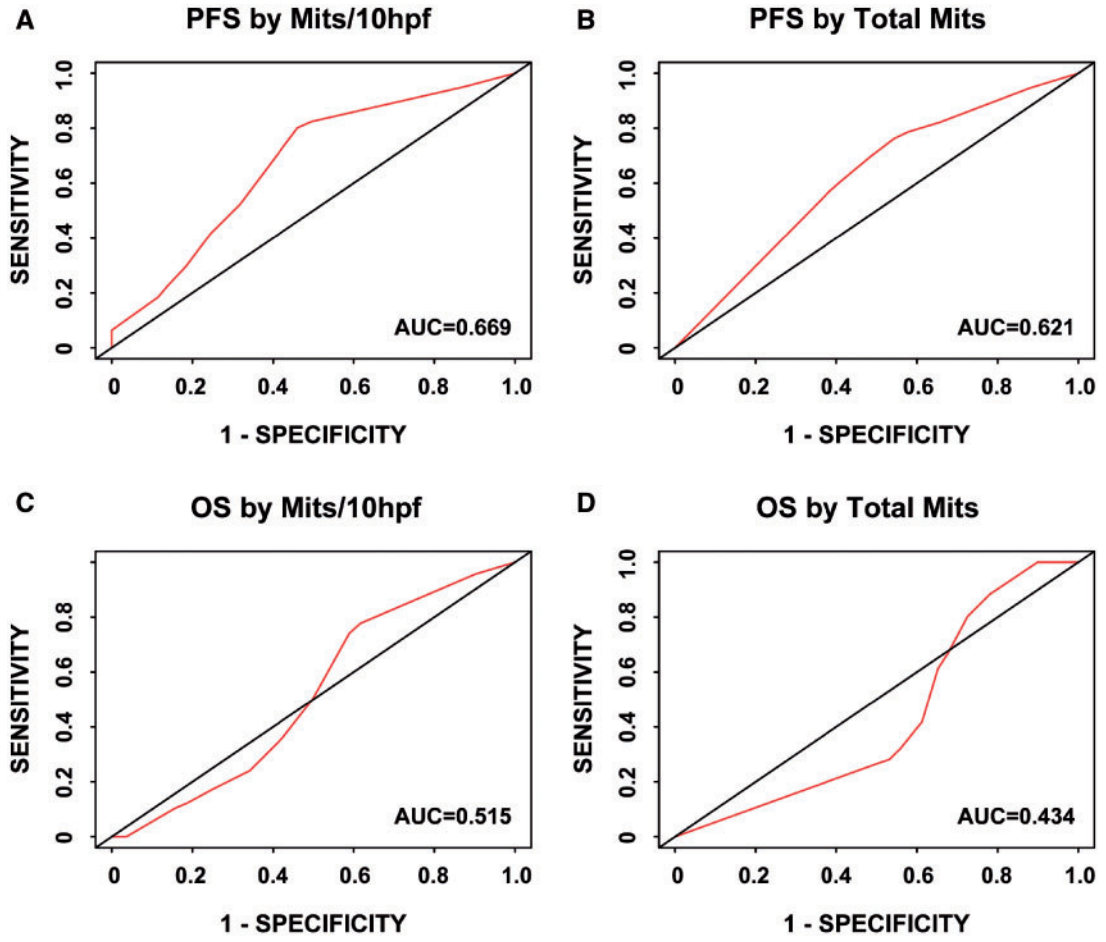

FIGURE 6. Receiver operating characteristic (ROC) estimation for censored survival data based on mitotic activity in IDH-mutant astrocytoma. ROC curves for progression-free survival (PFS) based on **(A)** maximum number of mitotic figures identified per 10 high-power fields (10 HPF) and **(B)** total mitotic figures. ROC curves for overall survival (OS) based on **(C)** maximum number of mitoses identified per 10 HPF and **(D)** total mitoses present in the pathology specimen.

TABLE. Multivariate Cox Proportional Hazards Model for Our Institutional Cohort of IDH-Mutant Astrocytomas

	PFS		OS	
	HR (95% CI)	**p Value**	**HR (95% CI)**	**p Value**
Age: ≥40 vs <40 years	1.49 (0.50–4.46)	0.48	5.47 (0.43–70.19)	0.19
KPS: 90%–100% vs 70%–80%	1.05 (0.37–3.04)	0.92	0.22 (0.03–1.67)	0.14
EOR: GTR vs STR	0.40 (0.15–1.03)	0.058	0.25 (0.02–3.03)	0.27
Total mitoses: ≥20 vs <20	1.49 (0.55–4.06)	0.43	5.86 (0.92–37.55)	0.062
MGMT: methylated vs unmethylated	0.25 (0.09–0.69)	**0.007**	0.38 (0.088–1.64)	0.19
CDKN2A: homozygous deletion vs no homozygous deletion	1.52 (0.44–5.24)	0.51	11.76 (1.98–71.43)	**0.007**
Chr14: loss vs no loss	8.00 (1.99–32.26)	**0.003**	2.07 (0.01–423.80)	0.79

HR, hazards ratio; PFS, progression-free survival; OS, overall survival; KPS, Karnofsky performance score; EOR, extent of resection; GTR, gross total resection; STR, subtotal resection; Chr14, chromosome 14.

results to suggest either qualitative or quantitative characterization of mitotic activity may be the wrong approach to grade IDH-mutant astrocytomas.

We have previously shown that chromosome 14 loss is a univariate poor prognostic indicator for PFS in this IDH-mutant astrocytoma cohort (5). In our current multivariate model, chromosome 14 loss is again identified as an aggressive molecular marker for PFS, and we have identified unmethylated *MGMT* promoter status as another independent correlate of poor PFS. Consistent with our previous report of *CDKN2A* homozygous

deletion as a univariate marker associated with worse OS (5), we identified *CDKN2A* homozygous deletion as the most relevant predictor of inferior OS among the investigated tumor feature in our current multivariate model.

Molecular alterations conferring a more aggressive ("higher-grade") clinical behavior are better understood in IDH-wildtype astrocytic gliomas. IDH-wildtype diffuse astrocytomas (WHO grade II) and anaplastic astrocytomas (WHO grade III) with certain molecular alterations (one or more of the following: 1—*EGFR* amplification; 2—*TERT* promoter mutation; 3—polysomy chromosome 7 co-occurring with monosomy chromosome 10) can be considered as "molecular glioblastoma, WHO grade IV" even in the absence of diagnostic histological features (i.e. necrosis, microvascular proliferation) traditionally required for the classification of glioblastoma (29). While the molecular pathways leading to aneuploidy in glioma are incompletely understood, there is evidence for *PDGFA* and *HOXA5* driving polysomy chromosome 7 in IDH-wildtype astrocytic gliomas (30, 31). Within the designation of histologically defined IDH-wildtype glioblastoma, a handful of SCNAs, including *CDK4*/*MDM2* coamplification, polysomy chromosome 1, and polysomy chromosome 19, have been demonstrated as having additional prognostic value beyond glioma histological grading (6, 32).

We know much less about gene and chromosomal level alterations in IDH-mutant astrocytomas, but it is expected that, like their IDH-wildtype counterparts, grading is likely to evolve and incorporate molecular features, specifically SCNAs, beyond IDH mutational status grading. Emerging evidence suggests that stratifying WHO grade II and III IDH-mutant astrocytic gliomas by SCNAs, such as *CDKN2A* deletion, is useful in predicting patient survival and may replace histological grading (3–6, 8–10). Determining SCNAs may also be more consistent and reproducible for identifying aggressive IDH-mutant diffuse astrocytic gliomas than current histopathological grading by mitotic activity alone, thereby reducing the interobserver variability (1, 16, 33, 34) and increasing uniformity in neuropathological diagnosis and clinical risk-stratification.

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